APPENDIX 4

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Regulation of Conceptus Adhesion by Endometrial CXC Chemokines During the Implantation Period in Sheep

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ABSTRACT To gain a better understanding of biochemical mechanisms of conceptus adhesion to the maternal endometrium in ruminant ungulates, the present study was performed to clarify roles of chemokines and extracellular matrix (ECM) components in the regulation of ovine blastocyst attachment to the endometrium. In addition to the chemokine, interferon-γ inducible protein 10 kDa (IP-10, CXCL10), the chemokine receptor, CXCR3, also recognizes two other chemokines; monokine induced by IFN-γ (MIG, CXCL9) and IFN-inducible T cell α chemoattractant (I-TAC, CXCL11). Similar to CXCL10, CXCL9, and CXCL11 were expressed in the uterus during the peri-implantation period, and CXCL9 mRNA expression was stimulated in endometrial explants from day 14 cyclic ewes by the addition of IFN-τ or IFN-γ. Without ECM components, conceptus cell adhesion was low on day 14 of gestation and exhibited a 2.5-fold increase on day 17; adhesiveness on day 20 was 1/10 of that on day 14. Among various ECM components examined, trophoblast adhesion was greatest when fibronectin was used. Although day 14 conceptuses did not show much adhesive activity to fibronectin, day 17 trophoblast, and day 20 chorionic membrane exhibited 2.3-fold and 50-fold increase, respectively, which was enhanced by treatment with CXCL9 or CXCL10. These results indicate that through endometrial fibronectin and chemokines, ovine conceptus cells gain the ability to attach to the endometrium during pre-implantation period; however, elucidation of molecular mechanisms by which the conceptus acquires the adhesive ability during this time period awaits further investigation. *Mol. Reprod. Dev. 73: 850–858, 2006.*

Key Words: CXC Chemokines; CXCL9; CXCL10; CXCL11; adhesion; trophoblast; ovine

INTRODUCTION

In mammals, a biochemical interaction between the conceptus and maternal system is required if pregnancy is to be established (Godkin et al., 1982; Roberts et al., 1992). In ruminant ungulates, the high incidence of embryonic loss occurring during the peri-implantation period (Roberts et al., 1992) may be caused by insufficient communication between the conceptus and maternal uterine endometrium. Interferon-tau (IFN-τ), produced by the conceptuses of ruminants (Imakawa et al., 1987; Stewart et al., 1987; Charpigny et al., 1988; Roberts et al., 1992), prevents corpus luteum regression (Spencer et al., 1995), resulting in the continued production of progesterone. This event has been regarded as the process of maternal recognition of pregnancy (Short, 1969). Effects of IFN-τ are mediated through type I IFN receptor located in the luminal epithelium of the endometrium (Godkin et al., 1984). IFN-τ can stimulate the transcription of uterine genes (Teixeira et al., 1997; Tuo et al., 1999; Emond et al., 2000) through the IFN-stimulated response element (ISRE) and interferon-γ-activated sequence (GAS) (Stewart et al., 2001), which are identical to the downstream events elicited by type II IFN, IFN-γ.

Quite recently, upregulation of a chemokine, IFN-γ inducible protein 10 kDa (IP-10, CXCL10), was found in the ruminant uterus during the peri-implantation period (Nagaoka et al., 2003a). Chemokines are classified into four groups (CXC, CC, C, and CX_3C) based on structural and genetic considerations, and mediate their functions such as attraction of specific leukocyte subsets through binding to seven-transmembrane-domain receptors coupled to G proteins (Moser and Ebert, 2003). CXCL10 is a member of the CXC chemokines and binds to CXC chemokine receptor 3 (CXCR3), which also binds two other CXC chemokines, monokine-induced by IFN-γ

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(MIG, CXCL9) and IFN-inducible T cell α chemoattractant (ITAC, CXCL11). Although the chemokine receptors often recognize more than one ligand and several chemokines can bind to multiple receptors, the CXC chemokine receptors are generally ligand specific (Opdenakker and Van Damme, 2004). Furthermore, some chemokines can enhance integrin-dependent adhesion in leukocytes and neutrophils (Gasperini et al., 1999; D'Ambrosio et al., 2002). In the uterus of mice and goats, chemokine receptors exist not only in the endometrium but also in trophoblast cells (Dominguez et al., 2003; Nagaoka et al., 2003b), suggesting that chemokines may regulate implantation processes such as conceptus adhesion to the maternal endometrium.

A specific interaction between the conceptus and endometrium has been reported during the implantation process (Lessey, 2002). The endometrium produces and secretes chemokines such as GCP2, IL-8, MCP-1, RANTES, and CXCL10 (Teixeira et al., 1997; Tuo et al., 1999; Caballero-Campo et al., 2002; Nagaoka et al., 2003b) during the early stage of gestation. Observations in which chemokine receptors were expressed in trophoblast cells (Dominguez et al., 2003; Nagaoka et al., 2003a) are sufficient to hypothesize that endometrial chemokines may regulate conceptus adhesion to the uterine epithelium. This study was undertaken to examine whether chemokines affect conceptus adhesion, and if adhesion of trophoblast cells to extracellular matrix (ECM) depends on the components of that matrix. Because endometrial CXCL10 production was regulated by conceptus IFN-τ (Nagaoka et al., 2003b), the expression of CXC chemokines (CXCL9, CXCL10, and CXCL11) that share the same CXCR3 receptor, was examined in ovine endometrium during the implantation period. Although CXCL9 and CXCL10 share the same receptor, their effectiveness in adhesive process often differs (Colvin et al., 2004). Examinations were then extended to determine whether or not the adhesiveness of ovine trophoblast cells is regulated through different ECM components and CXC chemokines, CXCL9 or CXCL10.

MATERIALS AND METHODS

Animals and Tissue Collections

All ewes were maintained at the U.S. Meat Animal Research Center, Clay Center, NE and care was in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, the U.S. Department of Agriculture. Animal care and estrous synchronization procedures were as described previously (Imakawa et al., 2002). Uteri were removed immediately after slaughter from cyclic ewes on day 14 (n = 6) and from pregnant ewes on days 14 (n = 16), 17 (n = 21), 20 (n = 12), 25 (n = 3), and 30 (n = 3) of gestation. Conceptuses at day 17 or younger of gestation were recovered from the uterus by flushing with 20 ml sterile PBS whereas day 20 or older conceptuses were removed from the uterus after longitudinal incision of the uterine horns. Two structures were collected separately from day 20 conceptuses; fluid filled allantochorion membrane (chorionic membrane) in which the embryo resides, and trophoblast tissues at distal ends of the chorionic membrane. For endometrial tissue collections, uterine horns were equally divided into three parts relative to the ovary; proximal, middle, and distal. Endometrial RNAs used in this study were extracted from the middle section of uterine horns. Except the middle section of endometrial tissues from day 14 cyclic ewes (n = 3) that were subjected to in vitro culture studies, endometrial tissues and uterine flushing media collected from cyclic and pregnant ewes were frozen immediately and stored at −80°C until use.

RT-PCR Analysis

Total RNA, isolated from frozen samples (n = 2 each from day 14 of estrous cycle, and from days 14, 17, and 20 of gestation) using Isogen (Nippon Gene, Tokyo, Japan), was reverse-transcribed using SuperScriptII (Invitrogen, Carlsbad, CA) and oligo-dT primers (20 μl reaction volume), and resulting cDNA (RT template) was kept at 4°C until use. Relative mRNA levels for chemokines and CXCR3 were analyzed using PCR amplification using oligonucleotide primers (Table 1). As a control, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was also amplified in each sample. Each reaction consisting of primer pairs for CXCL9/G3PDH, CXCL10/G3PDH, CXCL11/G3PDH, or CXCR3/G3PDH was run with RT template (1 μl) and AmpliTaq Gold (0.625 U; Roche Diagnostic, Mannheim, Germany) in a final volume of 25 μl. Ratio of primer pairs that gave each PCR product within the linear range had been determined: 5:1 for CXCL9: G3PDH, 6:1 for CXCL10: G3PDH, 3:1 for CXCL11: G3PDH, and 5:2 for CXCR3: G3PDH. PCR amplification consisted of 40 cycles at 94°C for 1 min, annealing at 53 (CXCL9), 50 (CXCL10), 55 (CXCL11), or 57°C (CXCR3) for 1 min, and extension at 72°C for 1 min. PCR products separated on 1.5% agarose gel and visualized with epthidium bromide were quantified using an image analysis system (ATTO Corporation, Tokyo, Japan) equipped with the Quantity One (v3.0 software; PDI, Inc., Huntington Station, NY). All PCR products were subcloned into pGEM-T easy vector (Promega, Madison, WI) and sequenced to verify nucleotide structures.

TABLE 1. Oligonucleotide Primers used for PCR Reactions

Name	Sequence of forward and reverse primer	Length (bp)
CXCL9	5′-cgctgttcctgcatcagcac-3′ 5′-actccgttcttcagtgtagc-3′	620
CXCL10	5′-cactcctcaactcttcaggc-3′ 5′-ccattccttttcattgtggc-3′	262
CXCL11	5′-ccactgtccccactgacttt-3′ 5′-ggcaatgacgaaggaggtta-3′	500
CXCR3	5′-gcatcagcttcgatcggtac-3′ 5′-gatgcgggcgtagcaatagg-3′	283
G3PDH	5′-atggggaaggtgaaggtcgg-3′ 5′-atcatattggaacatgtaaa-3′	150

Western Blot Analysis

Ovine uterine flushing was concentrated approximately 100-fold with a Centricon-3 (Millipore, Bedford, MA). The protein concentration of these samples was determined by Bradford's method (BioRad, Hercules, CA). The samples (30 μg/lane) were separated on a 15% reducing SDS–PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 5% (w/v) skim milk in Tris-buffered saline, the membranes were incubated with rabbit anti-human CXCL9 (PeproTech, London, UK), mouse anti-human CXCL10 (Genzyme, Cambridge, MA) or rabbit anti-human CXCL11 (PeproTech). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Amersham Biosciences, Piscataway, NJ) or donkey anti-rabbit IgG (Amersham Biosciences) was used as the secondary antibody. For visualization, SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) was used, and the signal was detected on an X-ray film (Kodak, Tokyo, Japan).

Endometrial Culture with IFNs

From endometrial tissues collected from day 14 cyclic ewes (n = 3), endometrial explants (300 mg) were cultured in a 35-mm culture dish (BD Falcon; BD, Franklin Lakes, NJ) containing Dulbecco's modified Eagle's medium (DMEM, 10 ml/culture dish, Sigma-Aldrich, St. Louis, MO) and antibiotics (40 units/ml penicillin and 40 μg/ml streptomycin, Sigma-Aldrich) under the condition previously described (Imakawa et al., 2002). Treatments added to the culture dishes at the initiation of cultures were: no cytokine (−), bovine IFN-τ (0.1, 10, or 1,000 ng/ml; Katakura Industries Co., Tokyo, Japan), or bovine IFN-γ (1,000 ng/ml, Katakura Industries Co.). Upon completion of the 24-hr incubation, culture media and tissues were collected separately and frozen immediately. Total RNA was extracted from these explants and used to evaluate the CXCL9 mRNA using Northern blot analysis.

Northern Blot Analysis

Anti-sense digoxigenin (DIG)-labeled cRNA probes for CXCL9 and G3PDH were generated from the CXCL9 and G3PDH cDNA constructs, respectively, by using T7 or SP6 RNA polymerase (Nagaoka et al., 2003a). Endometrial total RNAs (20 μg/lane) were separated by electrophoresis on a 1.0% agarose-formaldehyde gel and transferred to a nylon membrane (Biodyne-B; Pall, East Hills, NY). The membrane was prehybridized in hybridization buffer containing 5× NaCl/sodium citrate (SSC), 50% (v/v) deionized formamide, 50 mM sodium phosphate (pH 7.0), 7% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine, 50 μg/ml denatured salmon sperm DNA, and 2% blocking reagent (Roche Diagnostics) at 65°C for 1.5 hr, and then hybridized with cRNA probe (200 ng/ml) in fresh hybridization buffer at 60°C for 16 hr. After hybridization, the membrane was washed once with 2× SSC and 0.1% (w/v) SDS at 65°C for 30 min, washed twice with 0.1× SSC and 0.1% SDS at 65°C for 30 min, and then incubated with RNase-A (20 μg/ml) at 37°C for 1 hr. The membrane was incubated in blocking buffer (1% blocking reagent) at room temperature for 1 hr, followed by the addition of anti-DIG antibody (1:10,000 dilution, Roche Diagnostics). The membrane was washed three times in 100 mM maleic acid (pH 7.5), 150 mM NaCl, and 0.3% Tween 20 for 10 min each and finally rinsed in 100 mM Tris-HCl (pH 9.5) and 100 mM NaCl. The chemiluminescent reaction was performed in the latter solution containing CSPD reagent (1:100 dilution; Roche Diagnostics), and the membrane was exposed to X-ray film. Northern blots were quantified by scanning densitometry using an ES-2000 Epson-Scanner (Seiko Epson Corporation, Nagano, Japan) and Quantity One software (PDI).

Immunofluorescence

Tissue sections (10 μm) from three frozen conceptuses (day 17, three ewes) were mounted onto silan-coated slides and fixed in acetone (Nagaoka et al., 2003b). Non-specific binding was blocked by treatment with Block Ace (Dainippon-Sumitomo Pharma, Osaka, Japan) at room temperature for 1 hr; slides were then incubated with a mouse monoclonal antibody to human CXCR3 (10 μg/ml, R&D Systems, Inc., Minneapolis, MN) or normal mouse IgG (Sigma-Aldrich) at 4°C for 12 hr. After the primary antibody incubation, slides were treated with FITC-conjugated goat anti-mouse IgG (15 μg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at room temperature for 1 hr. Nuclei were stained with propidium iodide (2 μg/ml, Sigma-Aldrich). The slides were examined and digital images were captured on a confocal laser scanning microscope (FV300, OLYMPUS, Tokyo, Japan).

Cell Adhesion Assays

For the initial screening of potential ECM components to which trophoblast cells attach, 96-well plates (BD) were coated with 5 μg/ml of human collagen Type I (Southern Biotech, Birmingham, AL), human collagen Type IV (Southern Biotech), human plasma fibronectin (Chemicon, Temecula, CA), human fibronectin α-chymotryptic fragment 120K (Chemicon), human laminin (Sigma-Aldrich), bovine vitronectin (YAGAI, Yamagata, Japan), or 0.5 μg/ml bovine osteopontin (Genzyme) in PBS at room temperature for 2 hr or at 4°C overnight. Wells were washed three times with PBS and blocked at room temperature for 2 hr or at 4°C overnight with 1% BSA (Sigma-Aldrich) that had been heat inactivated at 70°C for 3 min.

Trophoblast tissues removed from day 17 pregnant uteri (n = 6) were placed in 2 × 50 ml plastic centrifuge tubes each containing 30 ml of DMEM with 0.2% collagenase (type IV, Sigma-Aldrich) and incubated at 37°C for 60 min. After centrifugation, dissociated trophoblast cells were resuspended in the culture media (DMEM) containing 10% FBS (Lot. S02829S1820, Bio-West, Nuaill, France) and allowed to recover at 37°C for 2 hr. They were then centrifuged and resuspended in Puck's Saline A (Sigma-Aldrich) at a concentration of

1×10^6 cells/ml to which Ca^{2+} and Mg^{2+} were added to the solution at final concentrations of 1.8 and 0.8 mM, respectively. These cells (1×10^5 cells/well) were added to substrate coated wells and incubated at 37°C for 1.5 hr. Cells were washed three times with PBS, fixed with 2% glutaraldehyde for 20 min, stained with 0.2% crystal violet in 2% ethanol for 15 min, washed with water, and solubilized with 1% SDS for 1 hr. Optical density of each well was read at 595 nm with a microplate reader (ARVO™ SX 1420 Multilabel Counter, PerkinElmer Life Sciences, Inc., Boston, MA). Samples were analyzed in duplicate or triplicate. Background binding of cells to wells was determined using BSA coated wells, and this value was subtracted from each well.

Results from various ECM component screenings revealed that fibronectin was a likely candidate for trophoblast adhesion. In the subsequent experiments, 24-well plates were coated with bovine plasma fibronectin (Wako Pure Chemical, Osaka, Japan) at a concentration of 10 μg/ml at room temperature for 2 hr. Following three washes with PBS, the plates were blocked with 1% BSA at room temperature for 30 min. New sets of conceptuses (day 14, n = 10; day 17, n = 9; day 20, n = 6) were dissociated in DMEM with 0.2% collagenase (type IV, Sigma-Aldrich) at 37°C for 30 min. These primary trophoblast cells (3×10^5 cells/well) were labeled with the intracellular fluorescent dye, 4 μM calcein-AM (Sigma-Aldrich) at 37°C for 30 min. Following three washes with DMEM, cells were incubated with 20 ng/ml of recombinant human CXCL9 (rhCXCL9, PeproTech) or recombinant caprine CXCL10 (rcCXCL10, Nagaoka et al., 2003b) with or without mouse anti-human CXCL9 antibody (30 μg, R&D Systems, Inc.), rabbit anti-rcCXCL10 antibody (30 μg, Nagaoka et al., 2003b), or normal rabbit IgG (30 μg, Sigma-Aldrich) at 37°C for 1 hr. The cells were then added to each well coated with fibronectin and the plates were incubated at 37°C for 1 hr. After incubation, unbound cells were removed by three washes with PBS, and the remaining cells were treated with 1% Triton X-100 and 10% ethanol in PBS. Fluorescence of cells was measured using a fluorescence reader (excitation filter 485 nm and emission filter 535 nm, PerkinElmer Life Sciences, Inc.). For the blocking experiments, rhCXCL9 and rcCXCL10 proteins had been preincubated at 37°C for 1 hr with 30 μg/ml of anti-hCXCL9 and anti-cCXCL10 antibodies, respectively, or control rabbit IgG (Sigma-Aldrich).

Statistical Analysis

Densitometry (optical density) measurements were subjected to least squares (LS) ANOVA, which employed the general linear models procedures of the Statistical Analysis System (version 6.0; SAS Institute, Cary, NC). The densitometry measurements from G3PDH PCR products and G3PDH mRNA were used as covariates for RT-PCR and Northern blot analyses, respectively. In adhesion assays, the number of cells, measurements of fluorescence, attached with a treatment was calculated as the number relative to the cells without any treatment, which was then analyzed statistically as aforementioned. The model used in the LS-ANOVA included treatment and replicate as sources of variation. The least square means (LSM) and SE illustrated in the figures were derived from this analysis.

RESULTS

Expression of CXC Chemokines in Endometrium and of CXCR3 in Trophoblast Cells

In RT-PCR analysis, CXCL9 and CXCL11 mRNAs were maximally expressed in the endometrium on day 17 of gestation whereas CXCL10 appeared highest on day 20 (Fig. 1A,B). To confirm these results, concentrated uterine flushing media were used to analyze protein contents of these chemokines. As seen in Figure 1C, CXCL9, CXCL10, and CXCL11 chemokines were found in the uterine flushing on day 17, indicating that all chemokines which share the same receptor, CXCL9, CXCL10, and CXCL11, existed in the ovine uteri during the implantation period. In the Northern blot analysis, total RNA isolated from ovine endometrium (day 14 cyclic, and days 14, 17, 20, 25, and 30 of gestation) was analyzed for the presence of CXCL9 mRNA (Fig. 2A). High levels of CXCL9 mRNA were detected on days 17, 20, and 25 of gestation.

Effect of IFN-γ or IFN-τ on Endometrial CXCL9 Expression

To investigate a potential factor(s) that may regulate CXCL9 mRNA expression in uteri, endometrial explants from day 14 cyclic ewes were cultured in vitro for 20 hr in the presence or absence of bovine IFN-τ, or bovine IFN-γ. Total RNAs extracted from cultured endometrial tissues were examined for CXCL9 mRNA by Northern blot analysis. Endometrial CXCL9 mRNA expression was stimulated ($P < 0.05$, Fig. 2B) with bovine IFN-τ (10 or 1,000 ng/ml) or bovine IFN-γ (1,000 ng/ml).

Presence of CXCR3 on the Conceptuses

Using RT-PCR analysis, expression of CXCR3 mRNA was then examined in RNAs extracted from days 14, 17, or 20 ovine conceptuses. Results presented in Figure 3A revealed that CXCR3 mRNA was expressed throughout the peri-implantation period. To find the cellular origin of CXCR3 receptor in ovine conceptus tissues, immunofluorecence analysis was performed using day 17 frozen conceptuses and anti-human CXCR3 antibody. Localization of CXCR3 receptor clearly differed from nuclei (Fig. 3B).

Trophoblast Adhesion to Various ECM Components

Due to the presence of various integrin ligands in the endometrium, we examined the binding ability of day 17 ovine trophoblast cells to several ECM components including human collagen Type I, human collagen Type IV, human fibronectin, human fibronectin 120 kDa fragment, human laminin, bovine vitronectin, and

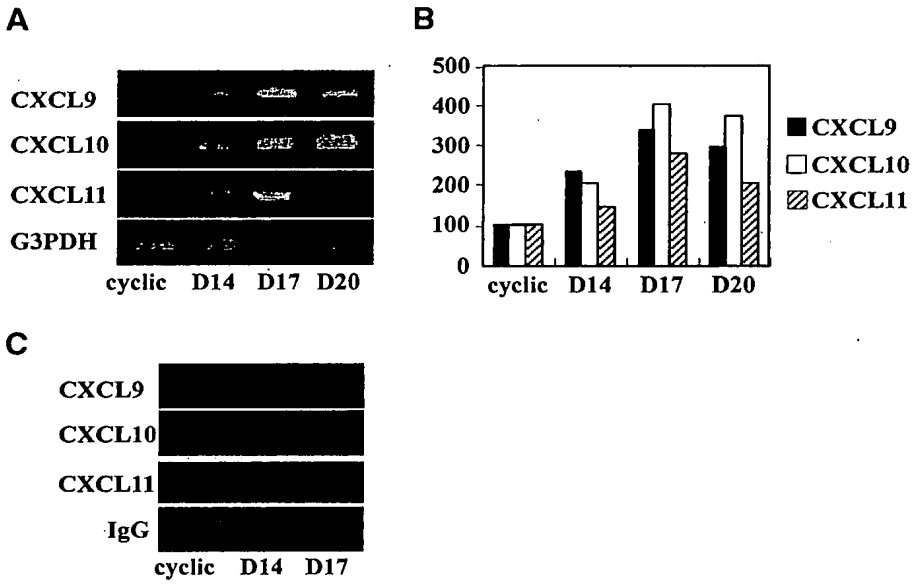

Fig. 1. Expression of CXC chemokines in endometrium and uterine flushing. **A**: RT-PCR analysis of CXCL9, CXCL10, and CXCL11 mRNAs in the ovine endometrium. Total RNA was extracted from ovine endometrium on day 14 cyclic and on days 14, 17, and 20 pregnant animals, and subjected to RT-PCR analysis with primers for ovine CXCL9, ovine CXCL10, human CXCL11, and ovine G3PDH mRNAs (Table 1). Two independent experiments were performed. **B**: Densitometric analysis of Northern blots of CXC chemokine mRNAs and G3PDH mRNA. **C**: Western blot analysis of CXCL9, CXCL10, and CXCL11 in the uterine flushing. Uterine flushing was obtained from day 14 cyclic, and days 14 and 17 pregnant animals. Samples were separated on a 15% SDS–PAGE gel under reducing conditions. A negative control for CXC chemokines is shown on the bottom. Two independent experiments were performed.

bovine osteopontin. As shown in Figure 4A, a major ligand for ovine trophoblast cells was fibronectin; without any chemokines, trophoblast cells could not bind to other ECM. After stimulation of trophoblast cells with CXC chemokines, these cells bound to collagen Type I and collagen Type IV; however, the adhesiveness of the cells was much less than to fibronectin (unpublished observations).

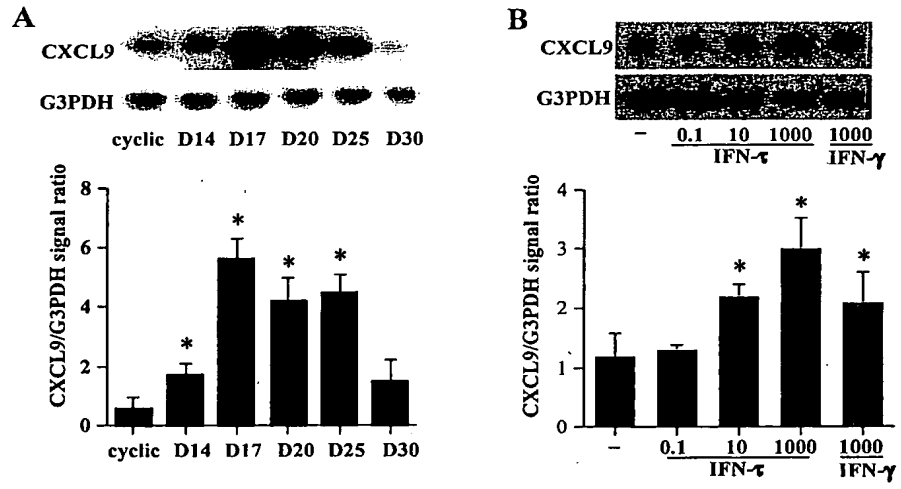

Fig. 2. Expression of CXCL9 mRNA in cyclic and pregnant sheep uteri. CXCL9 mRNA expression was examined by Northern blot analysis and is presented as CXCL9/G3PDH mRNA ratio. Total RNAs were extracted from day 14 (n = 3) cyclic and days 14 (n = 3), 17 (n = 3), 20 (n = 3), 25 (n = 3), and 30 (n = 3) pregnant animals. **A**: Upper: Representative Northern blot analysis. Lower: Densitometric analysis of Northern blots of CXCL9 mRNA. Bars represent LSM ± SE. An asterisk indicates a significant difference ($P < 0.01$) when compared to the value from day 14 cyclic uteri. Three independent experiments were performed. **B**: Expression of CXCL9 mRNA in the endometrium treated with IFN-τ or IFN-γ. CXCL9 mRNA expression was examined by Northern blot analysis, and was presented as CXCL9/G3PDH mRNA ratio. RNAs were extracted from the endometrium of day 14 cyclic ewes (n = 3), which had been treated with no IFN (–), bovine IFN-τ (0.1 ng/ml, 10 ng/ml, or 1000 ng/ml), or bovine IFN-γ (1000 ng/ml). Upper: Representative Northern blot analysis. Lower: Densitometric analysis of Northern blots. Bars represent LSM ± SE. An asterisk indicates a significant difference ($P < 0.05$) when compared with the value from endometrial explants cultured without cytokines. Three independent experiments were performed.

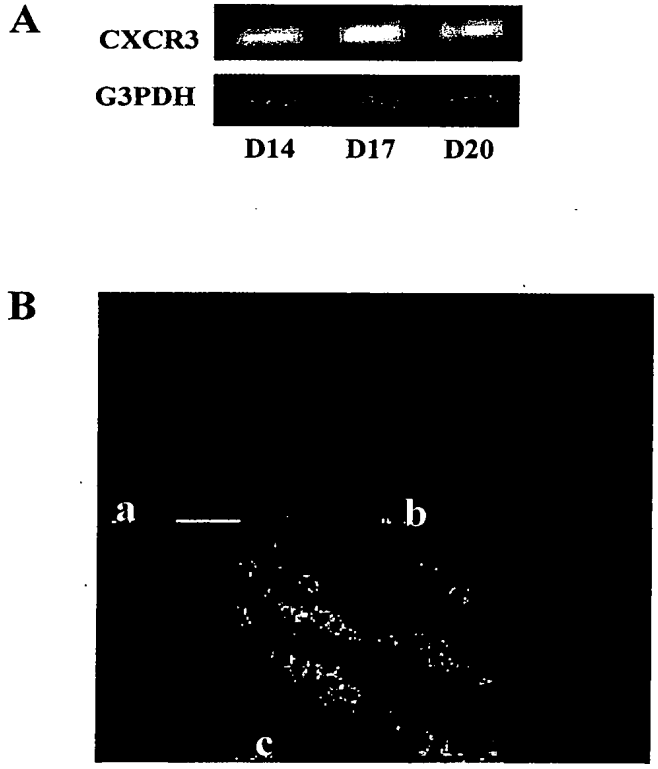

Fig. 3. Expression of CXCR3 in ovine trophoblast cells. **A**: RT-PCR analysis of CXCR3 mRNAs in days 14, 17, and 20 ovine conceptuses. Ovine trophoblasts from days 14 and 17, and membranes/trophoblasts from day 20 were recovered from the uterine lumen of pregnant ewes. Total RNA extracted was subjected to RT-PCR analysis with primers for ovine CXCR3 and G3PDH mRNAs (Table 1). **B**: Cellular localization of CXCR3 in the conceptus. Immunofluorescence microscopy was performed on day 17 ovine conceptuses using anti-human CXCR3 monoclonal antibody (a), and nuclei stained with propidium iodide were shown (b). c, combined fluorescence image resulting from anti-CXCR3 and propidium iodide was shown. Scale bar = 100 μm. Three independent experiments were performed for each treatment.

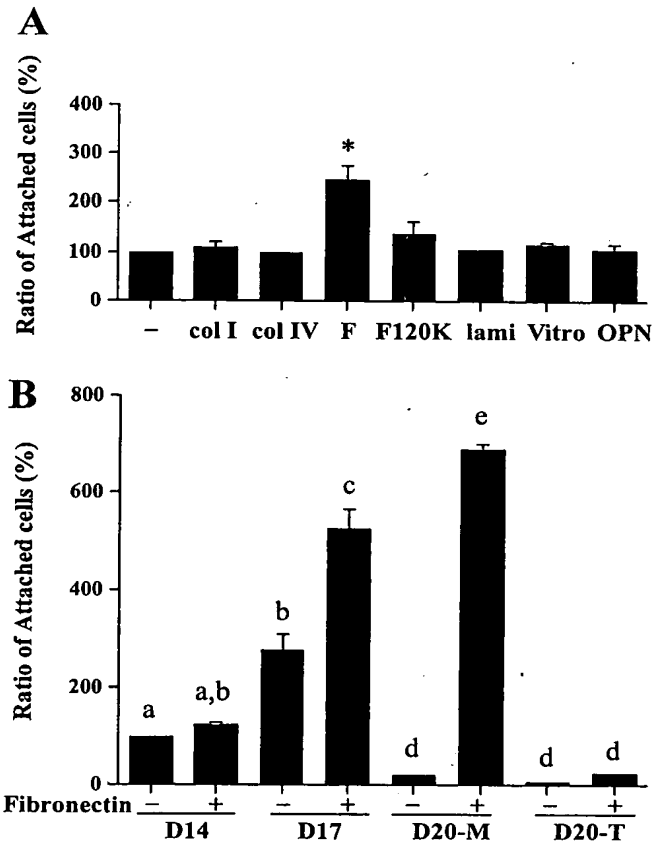

Fig. 4. Blastocyst adhesion to various ECM. **A**: Blastocyst adhesion to ECM components. Ovine trophoblast cells obtained from day 17 pregnant ewes (n = 6) were placed in plates coated with no substrate (–), human collagen Type I (col I), human collagen Type IV (col IV), human fibronectin (F), human fibronectin 120 (F120K), human laminin (lami), bovine vitronectin (vitro), or bovine osteopontin (OPN). Relative binding of trophoblast cells to different ligands, which exhibited some adhesive activity, is shown. Bars represent LSM ± SE and an asterisk indicates a significant difference ($P < 0.05$). Triplicate samples were examined for each treatment, and three independent experiments were performed. **B**: Conceptus adhesion to fibronectin. Adhesion of primary ovine trophoblast cells from days 14 (n = 10), 17 (n = 6), and 20 (n = 3) to the plates coated with (+) or without (–) fibronectin was assessed using adhesion assay. Note that dissociated cells from day 20 conceptuses tested separately were chorionic membranes (D20-M) and trophoblasts (D20-T). Bars represent LSM ± SE. Differences in the number of attached cells among days or fibronectin treatment were shown with a superscript ($P < 0.01$). Duplicate samples were examined for each treatment and three independent experiments were performed.

Effects of CXCL9 or CXCL10 on the Adhesion of Trophoblast Cells to Fibronectin

Calcein-AM labeled trophoblast cells were further evaluated for adhesiveness to fibronectin. Without fibronectin, trophoblast cell adhesion was low on day 14 of gestation and exhibited a 2.5-fold increase from day 14 to 17; adhesiveness on day 20 was 1/10 of that on day 14 (Fig. 4B). On day 17, trophoblast cells exhibited a 2.3-fold increase when fibronectin was placed in the primary cell cultures. On day 20 of gestation, approximately 50-fold increase in the adhesiveness was detected when fibronectin was placed into the dispersed chorionic membrane cells (Fig. 4B). Unlike cell preparations from day 20 chorionic membranes, day 20 trophoblast cells did not exhibit any adhesive activity in the presence or absence of fibronectin (Fig. 4B).

Because primary ovine conceptus cells from day 17 and 20 of gestation responded to fibronectin in the adhesion assay, these cells were then evaluated for adhesiveness to fibronectin in the presence or absence of the chemokine. Addition of CXCL9 or CXCL10 to day 17 trophoblast cell preparations further enhanced adhesive activity 30%–60% beyond the presence of fibronectin (Fig. 5A); chemokines were also effective (i.e., 50%–60% increase) in day 20 membrane cell preparations (Fig. 5B). Effects of chemokines were inhibited with the addition of respective antibodies (Fig. 5). It should be noted that day 20 membrane cell preparations exhibited approximately 30% more adhesive activity to fibronectin than those of day 17 trophoblast cell preparations (Fig. 4B). The degree of increase in the adhesive activity

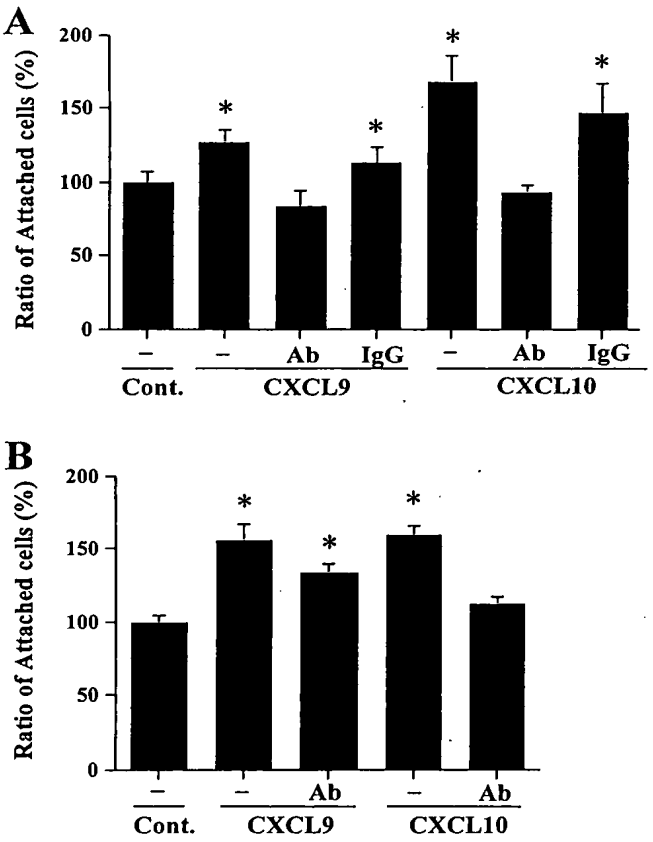

Fig. 5. Promotion of CXCL10/CXCL9 on the adhesion of days 17 and 20 ovine conceptus cells to fibronectin. Adhesion of primary ovine trophoblast cells (day 17, n = 3; day 20, n = 3) stimulated or not stimulated with CXCL9 or CXCL10 to the plates coated with fibronectin was assessed using adhesion assay. Blocking experiment was performed using the pretreatment of rhCXCL9 (20 ng/ml) and rcCXCL10 (20 ng/ml) with anti-human CXCL9 antibody (30 μg/ml) and anti-caprine CXCL10 antibody (30 μg/ml), respectively, or normal rabbit IgG (30 μg/ml), which was then added to cells. Cont.: No chemokine treatment. –: No addition of Antibody or IgG. Bars represent LSM ± SE, and asterisk(s) indicates a significant difference ($P < 0.01$). Duplicate samples were examined for each treatment, and three independent experiments were performed. A: Day 17 trophoblast. B: Day 20 chorionic membranes.

of membrane cells to fibronectin with a CXC chemokine on day 20 was greater than for day 17 trophoblast cell preparations. On the other hand, minimal effects of chemokines on adhesion were detected for day 14 and day 20 trophoblast cells (data not shown).

DISCUSSION

Chemokines were originally defined as proteins eliciting host defense mechanisms. It is now clear that their repertoire of functions extends well beyond this role. The major function of chemokines is to recruit immune cells such as leukocytes, lymphocytes, and macrophages to inflammatory regions (Red-Horse et al., 2001). Similarly, chemokines must play an integral role in the recruitment of various immune cells from peripheral blood vessels to the endometrium. In this respect, several chemokines found in the uterus, including MCP-1, MIP-1, IL-8, RANTES, and CXCL10, may function to recruit immune cells during the period of conceptus implantation into the maternal endometrium (Arici et al., 1998; Kyaw et al., 1998; Critchley et al., 1999; Caballero-Campo et al., 2002; Imakawa et al., 2005). Because more than 40 chemokines have been identified, chemokines other than the ones aforementioned may also be involved in these processes; however, little is known about which chemokines are expressed and how they affect implantation processes in ruminants. This study demonstrated that in addition to CXCL10, CXC chemokines CXCL9 and CXCL11, which share the same receptor, were expressed in ovine uteri during the implantation period. This finding is not surprising because differences in the expression of these CXC chemokine mRNAs were found in some other tissues and organs (Lacroix-Lamande et al., 2002; Salmaggi et al., 2002). Endometrial CXCL10, stimulated by conceptus IFN-τ, was found in monocytes localized in the subepithelial stroma (Nagaoka et al., 2003a); CXCL9 and CXCL11 mRNA were also localized in endometrial macrophages or monocytes (unpublished observations).

In this study, level of endometrial CXCL9 mRNA expression was enhanced by IFN-τ and IFN-γ (Fig. 2B), and the response was found to be similar to that for CXCL10 mRNA expression (Nagaoka et al., 2003a). CXCL9 mRNA expression is commonly induced by IFN-γ, a factor that activates the transcription of various genes through ISRE and GAS (Pine et al., 1994; Ohmori et al., 1997). The stimulation of uterine genes occurs via the same signaling cascade (Stewart et al., 2001). However, the observation that IFN-γ has not been found in the ovine uterus could exclude the possibility that endometrial CXCL9 expression is regulated by IFN-γ. In addition to IFN-τ, progesterone may also be involved in the regulation of endometrial CXCL9, CXCL10, and/or CXCL11 expression because other investigators have demonstrated that expression of chemokines GCP-2 and MCP-1/MCP-2 is regulated through both IFN-τ and progesterone (Teixeira et al., 1997; Asselin et al., 2001).

Endometrial expression of CXCL9, CXCL10, and CXCL11 (Imakawa et al., 2005) suggests the possibility that all of these chemokines are involved in recruitment of numerous leukocytes, lymphocytes, and/or monocytes to the uterus. Recently, mice carrying deletion mutants of the CXCL10 or CXCR3 gene have been produced (Hancock et al., 2000; Dufour et al., 2002), but neither group has shown any reproductive failure. This could be due to species' specificity in which other chemokines and their receptors with similar functions are alternatively expressed in the endometrium. In humans, unique distribution of chemokine CCR5 and CCR2B receptors, but not CXCR3, in the blastocyst has been found during the period of implantation (Dominguez et al., 2003). In this study, CXCL9, CXCL10, and CXCL11 mRNAs were found in the endometrium, and their CXCR3 receptor was found in trophoblasts during peri-adhesion period. Since a potential role of CXCL10 on trophoblast

adhesion was previously presented (Nagaoka et al., 2003b), these findings suggest that in addition to immune cell recruitment, interaction between chemokines and their receptors could also be required for conceptus adhesion to the endometrium.

Trophoblast cells obtained from uteri of day 14 pregnant sheep did not exhibit much adhesiveness to fibronectin. Moreover, their binding could not be enhanced by addition of CXC chemokines. This indicates that signaling cascade leading to integrin expression through CXCR3 (Nagaoka et al., 2003b) is still inactive in trophoblast cells on day 14. Without fibronectin, adhesiveness of trophoblast cells recovered from the uterus on day 17 of gestation was the highest; adhesion of chorionic membrane and trophoblast cells from day 20 conceptuses was minimal. These observations suggest that dynamic changes in the expression of adhesion molecules on the trophoblast/membrane cell surface may take place between days 14 and 17 of gestation, and become fibronectin specific toward day 20 of gestation. In fact, expression of fibronectin is very strong in endometrial epithelium and stroma cells in humans (Qin et al., 2003) as well as in baboons (Fazleabas et al., 1997). Additionally, several fibronectin receptors such as integrin $\alpha4\beta1$, $\alpha5\beta1$, $\alpha v\beta1$, and $\alpha v\beta3$ mRNAs were expressed on ovine trophoblast cells during the implantation period (Johnson et al., 2001; Kimmins et al., 2004). Different from fibronectin, other ECM are not favored by ovine trophoblast cells, which exhibited some affinity to collagens Type I and Type IV after chemokine treatment (unpublished observations). This could be caused by upregulation of integrin $\alpha11\beta1$, because it is the only collagen binding integrin identified in ovine trophoblast cells (unpublished observations). In support of this finding, MacIntyre et al. (2002) have shown that at least collagen Type IV is accumulated in the basal lamina and underlying subepithelial stroma during the period of bovine conceptus attachment to the endometrium. Although localization of collagens in the ovine endometrium has not been studied, these observations suggest that integrin $\alpha11\beta1$ could be functioning during adhesion process in sheep.

In summary, similar to CXCL10, chemokines CXCL9 and CXCL11 were also identified in the ovine uterus; CXCL9 expression was increased with the addition of IFN-τ. Although day 14 conceptuses did not show much adhesive activity to fibronectin, day 17 trophoblast, and day 20 chorionic membrane appeared to have acquired the ability to attach to fibronectin, which was enhanced by CXC chemokines. These results suggest that through endometrial chemokine expression, ovine conceptus cells gain the ability to attach to fibronectin; however, elucidation of molecular mechanisms by which the conceptus acquires the adhesive ability during this time period awaits further investigation.

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